Our Ref.: 410.018

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
ARAND et al	:	
PCT/FR00/01217	:	
Serial No.:	:	PCT Date: May 5, 2000
Filed: Concurrently Herewith	:	
For: EPOXIDE HYDROLASES OF ASPER-GILLUS ORIGIN	:	
	:	
	600 Third Avenue New York, NY 10016	

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Please amend this application as follows:

IN THE SPECIFICATION:

Page 1, before line 1, insert

--This application is a 371 of PCT/FR00/01217 filed May 5, 2000.--

IN THE CLAIMS:

Claim 2 (amended) A protein of Claim 1, comprising sequence SEQ ID NO : 2,

- or any sequence derived from sequence SEQ ID NO : 2, by substitution, suppression or addition of one or more amino acids,

and possessing epoxide hydrolase activity, the said derived sequence having a homology of at least about 40% with sequence SEQ ID NO : 2,

- or any fragment of sequence SEQ ID NO : 2, or of a sequence derived from the latter as defined above, and possessing epoxide hydrolase activity, the said fragment consisting of at least about 10 amino acids that are contiguous in the region delimited by the amino acids located in positions 1 and 339 of the sequence SEQ ID NO : 2.

Claim 3 (amended) A protein of Claim 1 corresponding to a fungal epoxide hydrolase in essentially pure form, as obtained by extraction and purification from cultures of cells of fungi of the *Aspergillus* species.

Claim 4 (amended) A protein of Claim 1 corresponding to the fungal epoxide hydrolase in essentially pure form represented by SEQ ID NO : 2, obtained by extraction and purification from cultures of cells of strains of the *Aspergillus niger* or *Aspergillus turingensis*.

Claim 5 (amended) A protein of Claim 1 corresponding to a recombinant fungal epoxide hydrolase, as obtained in essentially pure form by transformation of suitable host cells by means of vectors containing:

- the nucleotide sequence SEQ ID NO : 1 encoding the epoxide

hydrolase of SEQ ID NO : 2, or any sequence derived from SEQ ID NO : 1 by degeneration of the genetic code, and encoding the epoxide hydrolase represented by SEQ ID NO : 2,

- or any sequence derived from the sequence SEQ ID NO : 1, by substitution, suppression or addition of at least one nucleotide, and coding for an enzyme possessing epoxide hydrolase activity, the said derived sequence having a homology of at least about 45% with the sequence SEQ ID NO : 1,

- or any fragment of the sequence SEQ ID NO : 1, or of a sequence derived from the latter as defined above, and coding for an enzyme possessing epoxide hydrolase activity, the said fragment consisting of at least about 20 nucleotides that are contiguous in the region delimited by the nucleotides located in positions 1 and 1197 of the sequence SEQ ID NO : 1.

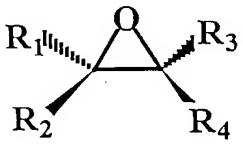
Claim 6 (amended) A protein of Claim 5, corresponding to the fungal recombinant epoxide hydrolase represented by SEQ ID NO : 2 as obtained by transformation of suitable host cells by vectors containing the nucleotide sequence SEQ ID NO : 1, or any sequence derived from SEQ ID NO : 1 by degeneration of the genetic code, and encoding the epoxide hydrolase represented by SEQ ID NO : 2.

Claim 7 (amended) A nucleotide sequence encoding a protein of fungal origin with epoxide hydrolase activity as defined by Claim 1.

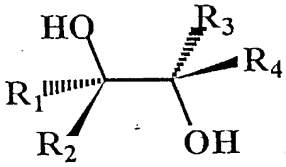
Claim 9 (amended) A vector, especially a plasmid, containing a nucleotide sequence according to Claim 7.

Claim 10 (amended) A host cell selected from the group consisting of bacteria, viruses, yeasts, fungi, plants and mammalian cells, the said host cell being transformed by a vector of Claim 9 so that its genome contains a nucleotide sequence of encoding a protein of fungal origin with epoxide hydrolase activity.

Claim 12 (amended) A method of preparation of epoxides and/or of enantiomerically pure diols respectively of the following formulae



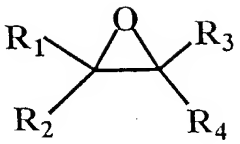
II



III

in which R^1, R^2, R^3 and R^4 represent any group characteristic of pharmaceutical and plant-protection compounds, or of specific optical materials corresponding to the said epoxides or vicinal diols,

comprising treating a mixture of diastereoisomeric epoxides, or of a chiral epoxide in racemic form, or of a prochiral epoxide of the formula:



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with a protein with epoxide hydrolase activity of Claim 1 or with the host cells of Claim 10 expressing a protein with epoxide hydrolase activity of Claim 1, which leads to the production of:

- a mixture of the aforementioned compounds of formulae (II) and (III), and optionally separating the said compounds of formulae (II) and (III) by an additional stage of purification,
- or of just the aforementioned compound of formula (III).

Claim 13 (amended) A method of preparation of a protein with recombinant epoxide hydrolase activity of Claim 5 comprising transforming host cells selected from the group consisting of bacteria, viruses, yeasts, fungi, plants and mammalian cells with a vector of Claim 9, and purifying the recombinant epoxide hydrolase produced by the said cells.

Claim 14 (amended) A method of preparation of a protein with epoxide hydrolase activity in essentially pure form of Claim 3 comprising:

- extracting the enzyme from cellular cultures of fungi by crushing the fungus using a press, followed by low-speed

centrifugation, recovery of the supernatant, and optionally, concentration of the same,

- and purifying the enzyme from the extract obtained in the preceding stage by successive passages through columns of DEAE-Sepharose, Phenyl-Sepharose, Mono Q and Superose 12.

Cancel claim 11 and add the following claim:

--15. In the process of preparation of epoxides or enantiomerically pure vicinal diols with enzymatic biocatalysts, the improvement comprising using as the biocatalyst a protein of claim 1 with epoxide hydrolase activity.--

REMARKS

The amendment is submitted to insert reference to the PCT application, remove multiple dependency from the claims and to write claims in the American fashion.

Respectfully submitted,
BIERMAN, MUSERLIAN AND LUCAS

Charles A. Muserlian, #19,683
Attorney for Applicant(s)
Tel. # (212) 661-8000

CAM:sd
Enclosures: Marked-Up Version of Claim
Return Receipt Postcard

410.018

MARKED-UP VERSION OF CLAIMS

1. A protein of fungal origin having epoxide hydrolase activity, ~~such as is~~ as obtained in essentially pure form by extraction from cells of fungi, or by culture of host cells transformed by a nucleotide sequence coding for the aforementioned fungal protein, or protein derived by substitution, suppression or addition of one or more amino acids of the aforementioned protein of fungal origin and possessing epoxide hydrolase activity.

2. A protein ~~according to~~ of Claim 1, ~~characterized in that it comprises:~~ comprising

~~—~~ ~~the~~ sequence SEQ ID NO : 2,

– or any sequence derived from ~~the~~ sequence SEQ ID NO : 2, ~~especially~~ by substitution, suppression or addition of one or more amino acids, and possessing epoxide hydrolase activity, the said derived sequence ~~preferably~~ having a homology of at least about 40% with ~~the~~ sequence SEQ ID NO : 2,

– or any fragment of ~~the~~ sequence SEQ ID NO : 2, or of a sequence derived from the latter as defined above, and possessing epoxide hydrolase activity, the said fragment ~~preferably~~ consisting of at least about 10 amino acids that are contiguous in the region delimited by the amino acids located in positions 1 and 339 of the sequence SEQ ID NO : 2.

3. A protein ~~according to~~ of Claim 1 ~~or 2, characterized in that it corresponds~~ corresponding to a fungal epoxide hydrolase in essentially pure form, ~~such~~ as ~~is~~ obtained by extraction and purification from cultures of cells of fungi of the *Aspergillus* species.

4. A protein ~~according to one~~ of ~~the~~ Claims 1 ~~to 3, characterized in that it corresponds~~ corresponding to the fungal epoxide hydrolase in essentially pure form represented by SEQ ID NO : 2, ~~such as is~~ obtained by extraction and purification from cultures of cells of strains of *Aspergillus niger* or of *Aspergillus turingensis*.

5. A protein ~~according~~ of to Claim 1 ~~or 2, characterized in that it corresponds~~ corresponding to a recombinant fungal epoxide hydrolase, ~~such~~ as ~~is~~ obtained in essentially pure form by transformation of suitable host cells by means of vectors containing:

– the nucleotide sequence SEQ ID NO : 1 encoding the epoxide hydrolase ~~represented by~~ SEQ ID NO : 2, or any sequence derived from SEQ ID NO : 1 by degeneration of the genetic code, and encoding the epoxide hydrolase represented by SEQ ID NO : 2,

– or any sequence derived from the sequence SEQ ID NO : 1, ~~especially~~ by substitution, suppression or addition of at least ~~one or more~~ nucleotides, and coding for an enzyme possessing epoxide hydrolase activity, the said derived sequence ~~preferably~~ having a homology of at least about 45% with the sequence SEQ ID NO : 1,

– or any fragment of the sequence SEQ ID NO : 1, or of a sequence derived from the latter as defined above, and coding for an enzyme possessing epoxide hydrolase activity, the said fragment ~~preferably~~ consisting of at least about 20 nucleotides that are contiguous in the region delimited by the nucleotides located in positions 1 and 1197 of the sequence SEQ ID NO : 1.

6. A protein ~~according~~ to Claim 5, ~~characterized in that it corresponds~~ corresponding to the fungal recombinant epoxide hydrolase represented by SEQ ID NO : 2, ~~such~~ as ~~is~~ obtained by transformation of suitable host cells by ~~means of~~ vectors containing the nucleotide sequence SEQ ID NO : 1, or any sequence derived from SEQ ID NO : 1 by degeneration of the genetic code, and encoding the epoxide hydrolase represented by SEQ ID NO : 2.

7. A nucleotide sequence encoding a protein of fungal origin with epoxide hydrolase activity ~~such~~ as ~~is~~ defined by ~~one of the~~ Claims 1 ~~to 6~~.

8. A nucleotide sequence according to Claim 7, characterized in that it comprises:

– the sequence represented by SEQ ID NO : 1 encoding the epoxide hydrolase represented by SEQ ID NO : 2,

– or any sequence derived from the sequence SEQ ID NO : 1 by degeneration of the genetic code, and encoding the epoxide hydrolase represented by SEQ ID NO : 2,

– or any sequence derived from the sequence SEQ ID NO : 1, especially by substitution, suppression or addition of one or more nucleotides, and coding for an enzyme possessing epoxide hydrolase activity, the said derived sequence preferably having a homology of at least about 45% with the sequence SEQ ID NO : 1,

– or any fragment of the sequence SEQ ID NO : 1, or of a sequence derived from the latter as defined above, and coding for an enzyme possessing epoxide hydrolase activity, the said fragment preferably consisting of at least about 20 nucleotides that are contiguous in the region delimited by the nucleotides located in positions 1 and 1197 of the sequence SEQ ID NO : 1,

– or any complementary nucleotide sequence of the aforementioned sequences or fragments,

– or any nucleotide sequence coding for an enzyme possessing epoxide hydrolase activity, and capable of hybridization with one of the aforementioned sequences or fragments,

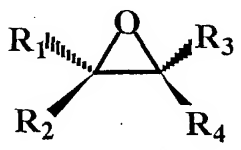
the aforementioned sequences or fragments being of single-stranded or double-stranded form.

9. A vector, especially a plasmid, containing a nucleotide sequence according to Claim 7 ~~or 8~~.

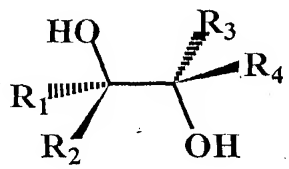
10. A host cell, ~~in particular chosen from~~ selected from the group consisting of bacteria, viruses, yeasts, fungi, plants ~~or~~ and mammalian cells, the said host cell being transformed, ~~especially~~ by ~~means of~~ a vector ~~according to~~ of Claim 9, ~~in such a way~~ so that its genome contains a nucleotide sequence ~~according to Claim 7 or 8~~ encoding a protein of fungal origin with epoxide hydrolase activity.

11. The use of proteins with epoxide hydrolase activity ~~defined in~~ one of the Claims 1 to 6, as ~~enzymatic~~ biocatalysts in the ~~implementation~~ of methods of preparation of epoxides or of ~~enantiomerically~~ pure vicinal diols, especially in the pharmaceutical ~~and plant~~-protection field, or in the field of ~~manufacture~~ of specific ~~optical~~ materials.

12. A method of preparation of epoxides and/or of enantiomerically pure diols respectively of the following formulae ~~(II) and (III)~~



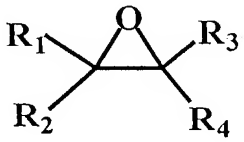
II



III

in which R1, R2, R3 and R4 represent any groups, ~~especially groups that are characteristic of pharmaceutical and plant-protection compounds, or of specific optical materials corresponding to the said epoxides or vicinal diols,~~

~~the said method~~ comprising ~~a stage of treatment of~~ a mixture of diastereoisomeric epoxides, or of a chiral epoxide in racemic form, or of a prochiral epoxide of the ~~following~~ formula ~~(I)~~:



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with a protein with epoxide hydrolase activity ~~according to one~~ of ~~the~~ Claims 1 to ~~6~~, or with the host cells ~~according to~~ Claim 10 expressing a protein with epoxide hydrolase activity ~~according to one~~ of ~~the~~ Claims 1 ~~to 6~~, which leads to the production of:

– a mixture of the aforementioned compounds of formulae (II) and (III), ~~it being possible, if necessary, for~~ the said compounds of formulae (II) and (III) ~~to be separated~~ by an additional stage of purification,

– or of just the aforementioned compound of formula (III).

13. A method of preparation of a protein with recombinant epoxide hydrolase activity ~~according to~~ Claim 5 ~~or 6, characterized in that it~~ comprises ~~a stage of~~ transformation ~~of~~ host cells ~~preferably chosen from the~~ bacteria, viruses, yeasts, fungi, plants ~~or~~ mammalian cells, with a vector ~~according to~~ Claim 9, and ~~a stage of~~ purification ~~of~~ the recombinant epoxide hydrolase produced by the said cells.

14. A method of preparation of a protein with epoxide hydrolase activity in essentially pure form ~~according to~~ Claim 3 ~~or 4, the said method~~ comprising:

– ~~a stage of~~ extraction ~~of~~ the enzyme from cellular cultures of fungi, ~~such as fungi of the *Aspergillus* species, especially~~ by crushing the fungus using a press, followed by ~~a stage of~~ low-speed centrifugation, recovery of the supernatant, and, ~~if required~~, concentration of the same,

~~a stage of~~ purification of the enzyme from the extract obtained in the preceding stage, ~~especially~~ by successive passages through columns of DEAE-Sepharose, Phenyl-Sepharose, Mono Q and Superose 12.

410.018

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: :
ARAND et al :
Serial No.: 10/009,030 :
Filed: November 2, 2001 :
For: EPOXIDE...ORIGIN :

600 Third Avenue
New York N.Y. 10016
December 20, 2002

RESPONSE

Asst. Commissioner for Patents
Washington, D.C. 20231

Sir:

Responsive to the formalities letter dated November 21, 2002, please amend this application as follows:

IN THE SPECIFICATION:

Page 17, rewrite lines 12 and 13 as follows:

--(MA2265'-ATGCGATCGGACTGCTGGACA-3' and SEQ ID NO: 3

MA2275'-CGCGGGCAATCCACACCTAC-3') SEQ ID NO: 4--

Page 17, rewrite lines 25 and 26 as follows:

--(MA2905'-cggaattccATGgTCACTGGAGGAGCAATAATTAG-3' AND SEQ ID No:5

MA2915'-ttgaatTCCTACTTCTGCCACAC-3' SEQ ID No: 6; the residues in capital letters are--

Page 18, rewrite line 5 as follows:

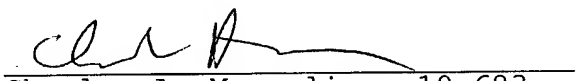
--5'gctgaattcacATGTCCGCTCCGTTCGCCAAG-3') SEQ ID No: 7--

Page 18, rewrite line 11 as follows:

--(5'-CCATGGGAATTCTCGAGATCTAAGCTTATGCATCAGCTGCATGG-3') SEQ ID No: 8--

Applicants are submitting herewith a paper copy of the sequence listing as well as an amendment directing its entry into the specification as well as a computer readable form of the sequence listing. The contents of the paper and computer readable form are the same and where applicable, include no new matter as required by 37 CFR 1.821(e), 1.82(f), 1.821(g), 1.825(b) or 1.825(d). Also enclosed is a copy of the notice of defective response. It is believed that the filing of the application is now complete and it is requested that the same be forwarded to the group for examination in due course.

Respectfully submitted,
Bierman, Muserlian and Lucas



Charles A. Muserlian, 19,683
Attorney for Applicants
Tel.# (212) 661-8000

CAM:ds
Enclosures

410.018

MARKED UP COPY OF SPECIFICATION SHOWING CHANGES MADE

Page 17, rewrite lines 12 and 13 as follows:

--(MA2265'-ATGCGATCGGACTGCTGGACA-3' and SEQ ID NO: 3

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Page 18, rewrite line 11 as follows:

--(5'-CCATGGGAATTCTCGAGATCTAAGCTTATGCATCAGCTGCATGG-3') SEQ ID No: 8--

2) Cloning of the gene of the EH of *Aspergillus* and of cDNA by the polymerase chain amplification technique (polymerase chain reaction, PCR)

The reverse PCR for amplification of the gene of the *Aspergillus* EH was effected according to the following scheme: 500 ng of genomic DNA is digested with a suitable restriction enzyme (most of the successful results are obtained with BamHI or CfoI) and are recovered by precipitation with ethanol after extraction with phenol/chloroform mixture. Of this 500 ng, 100 ng is circularized by ligation with DNA ligase T4 (Life Technologies) in a volume of 20 µL in the conditions specified by the supplier. One microlitre of the resulting preparation was amplified by PCR effected for 30 cycles (1 min 94°C, 1 min 60°C, 3 min 72°C) with a DNA polymerase Taq (Perkin Elmer) in the standard reaction conditions recommended by the supplier. The primers used

(MA226 5'-ATGCGATCGGACTGCTGGACA-3' and SEQ ID No: 3

MA227 5'-CGCGGGCAATCCACACCTAC-3') SEQ ID No: 4

are deducted from the sequence of a genomic fragment obtained previously. An XhoI restriction site located between the two priming sites in the genomic sequence is used optionally for relinearizing the circular DNA before the reverse PCR, in order to suppress the torsional stress and so improve the efficiency of initial amplification of the genomic support. The PCR products are separated by electrophoresis on agarose gel and the specific amplicons of the EH of *Aspergillus* are identified by immunotransfer according to the Southern technique using the aforementioned genomic fragment as a probe. The fragments of *Aspergillus* EH gene identified in this way are purified by electrophoresis on agarose gel using the Quiaex kit (Qiagen), and cloned in the pGEM-T vector (Promega) for sequence analyses by the chain termination method.

On the basis of the information obtained from the sequence, 2 primers

(MA290 5'-cggaattccATGgTCACTGGAGGAGCAATAATTAG-3' and SEQ ID No: 5
MA291 5'-ttgaatTCCCTACTTCTGCCACAC-3' SEQ ID No: 6; the residues in capital letters are
complementary to the support sequence) surrounding the region encoding the protein of the EH gene are deducted and used for amplifying the respective fragments of the genomic DNA and for reverse-transcribing the mRNA with high fidelity DNA polymerase Pfu ("Stratagene") for 40 cycles (1 min 94°C, 1 min 50°C, 6 min 72°C). The resulting DNA fragments are digested with EcoRI and inserted in pUC19 (New England Biolabs) for final sequence analysis.

3) Expression, purification and analysis of recombinant epoxide hydrolase

For recombinant expression in E. coli, the cDNA fragment of the epoxide hydrolase of *Aspergillus* is amplified with a DNA polymerase Pfu using the primer MA291 (see above) and the primer MA318

(5'gctgaattcacATGTCCGCTCCGTTCGCCAAG-3') SEQ ID No: 7

in order to introduce an AflIII NcoI-compatible recognition site (underlined in primer MA318) in the probable initiation codon of the epoxide hydrolase gene of *Aspergillus* which was revealed by sequence analysis.

The pGEF+ bacterial expression vector is modified by introducing a multiple cutting site

(5'-CCATGGGAATTCTCGAGATCTAAGCTTATGCATCAGCTGCATGG-3') SEQ ID No 8

in the NcoI site that contains the starting codon of the pGEF+ vector in the context adapted to a ribosome binding site, upstream of the promoter of the RNA polymerase T7. The resulting plasmid is called pGEF II hereinafter. The PCR fragment AflII/Eco RI of the EH of *Aspergillus* is ligated in the NcoI/Eco RI site of pGEF II to produce the pGEF Asp EH" expression construction. The E. coli strain BL21 (DE 3) (Novagen) is transformed with pGEF Asp EH and put in the LB medium at 30°C. In late exponential phase, induction of expression of the recombinant protein is effected by adding isopropyl-β-thiogalactoside (100 μM). After two hours, the bacteria are collected by centrifugation, resuspended in 0.02 volumes of culture of the STE buffer (Tris-HCl, 10 mM, sodium chloride 100 mM, ethylenediamine tetraacetic acid 1 mM, pH 7.4) and stored at -70°C. Enzymatic activity is determined by converting the R enantiomer of *para*-nitrostyrene oxide to the corresponding diol. The reaction is carried out at a substrate concentration of 880 μM in 500 μL STE at 37°C for 30 min, in the presence of 10 μL of acetonitrile which is used as solvent of *para*-nitrostyrene oxide.

The conversion reaction is terminated by extraction of the substrate with an equal volume of chloroform. In these conditions, more than 99.9% of the substrate is extracted in the organic phase and 60% of the diol is recovered in the aqueous phase.

The conversion substrate is quantified by adding 400 μL of supernatant to 800 μL of water and reading the optical density at 277 nM, with the molar extinction coefficient of the product being $9.1 \times 10^3\ M^{-1}\ cm^{-1}$. The epoxide hydrolase of *Aspergillus* is purified to homogeneity by a three-stage procedure, according to the method described above.